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Crosslinking Decreases the Hemocompatibility of Decellularized, Porcine Small Intestinal Submucosa

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Abstract

Decellularized tissues have been widely used as scaffolds for biomedical applications due to their presentation of adhesion peptide sequences and growth factors, which facilitate integration with surrounding tissue. One of the most commonly used decellularized tissue is derived from porcine small intestinal submucosa (SIS). In some applications, SIS is crosslinked to modulate the mechanical properties or degradation rate of the scaffold. Despite the widespread use of SIS, there has been no mechanistic characterization of blood reactions with SIS, nor how crosslinking affects these reactions. Therefore, we characterized the effect of SIS and carbodiimide-crosslinked SIS (cSIS) on plasma coagulation, including targeted assessments of the intrinsic and extrinsic coagulation pathways, and thrombus formation using flowing whole blood. SIS inhibited plasma coagulation initiated by recalcification, as well as low concentrations of thrombin or tissue factor. SIS prolonged the activated partial thromboplastin time by 14.3 ± 1.54 sec, indicating inhibition of the intrinsic coagulation pathway. Carbodiimide crosslinking abrogated all anticoagulant effects of SIS, as did heparinase I and III treatment, suggesting heparin and heparan sulfate are predominantly responsible for SIS anticoagulant effects. Inhibiting contact activation of the intrinsic pathway prevented cSIS-mediated coagulation. When tubular SIS devices were connected to a nonhuman primate arteriovenous shunt loop, which enables whole blood to flow across devices without the use of anticoagulants, SIS demonstrated remarkably limited platelet accumulation and fibrinogen incorporation, while cSIS initiated significantly higher platelet and fibrinogen accumulation. These results demonstrate that SIS is a thromboresistant material and crosslinking markedly reduces the hemocompatibility of SIS.

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8. Disclosure

The authors have no conflicts of interest to declare.

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Keywords

SIS (small intestinal submucosa); Hemocompatibility; Coagulation; Fibrinogen; Platelets

1. Introduction

The extracellular matrix presents cells with a variety of topographical and biochemical cues that help coordinate proper cellular function.[1,2] To recapitulate these signals, researchers have utilized decellularized extracellular matrices as a scaffold for a variety of biomedical and tissue engineering applications.[3,4] One of the most-studied decellularized matrices is derived from porcine small intestinal submucosa (SIS).[5,6] SIS is primarily composed of a network of collagens, glycosaminoglycans (GAGs), and growth factors including FGF-2 and VEGF.[7–9] The bioactive nature of the SIS material generally confers a favorable host response, characterized by cellular infiltration, tissue ingrowth, and limited pro-inflammatory M1-polarized macrophages.[10] Despite being derived from porcine intestine, SIS does not generally elicit any substantial adverse adaptive immune response.[11] Due to these advantageous characteristics, SIS has been successfully used clinically for numerous surgical procedures including dural grafts[12], hernia repair[13] and abdominal wall reconstruction.[14] In some applications, SIS is crosslinked to increase the mechanical robustness or reduce the degradation rate of the material.[15,16] However, crosslinking SIS can result in a chronic inflammatory response and fibrosis when implanted into an abdominal wall defect.[10] Thus, the inflammatory response and remodeling of SIS implanted in soft tissues can be fundamentally altered by crosslinking the material.

A number of groups have sought to use SIS for cardiovascular applications including vascular grafting[17,18], stent coverings[19,20] and venous valve replacement.[21] In these applications, SIS interacts predominantly with blood rather than soft tissues alone, and must serve as a non-thrombogenic surface with minimal activation of platelets or coagulation factors. Studies using SIS for cardiovascular applications have generally demonstrated low rates of thrombosis that were similar to autologous saphenous vein[18] and ePTFE.[22] However, unlike the thorough characterization of the inflammatory and immune responses to SIS, the thrombotic response has not been well-studied. Furthermore, the effect of crosslinking on the hemocompatibility of SIS has not been addressed.

The thrombogenicity of a biomaterial can be characterized using experimental procedures that vary greatly in complexity and similarity to *in vivo* conditions.[23,24] Purified systems that measure the activity of a defined subset of coagulation factors provide straightforward information on biomaterial thrombogenicity and are useful for providing mechanistic insight into biomaterial-associated thrombosis. However, these results may not translate to *in vivo* performance due to the highly-interconnected regulatory pathways of coagulation pathways as well as the blood flow-dependent transport of coagulation factors to and from the material. Methods that study biomaterial hemocompatibility using whole blood, particularly at physiological flow rates, provide the best indication of *in vivo* thrombogenicity, though these systems can confound the roles of individual blood components. To characterize SIS hemocompatibility, we utilized an array of *in vitro* coagulation assays using either purified solutions of coagulation factors or platelet-poor plasma to determine the effect of SIS on the

intrinsic and extrinsic coagulation pathways. Additionally, an *ex vivo* arteriovenous shunt loop was used to characterize thrombus formation using flowing whole blood. These studies provide a multi-faceted characterization of coagulation factor activation, plasma coagulation, and thrombus formation on SIS, and also determine how crosslinking SIS affects these thrombotic processes.

2. Materials and Methods

2.1 SIS preparation

Sheets of sterile, vacuum-pressed SIS were generously provided by Cook Biotech. Unless otherwise specified, SIS was cut into discs to fit into standard 96-well plates using 5 mm biopsy punches for all studies. Crosslinking SIS was performed according to the general crosslinking instructions provided by the carbodiimide manufacturer (Pierce), and similarly to other crosslinking procedures for SIS[25] and collagen-based biomaterials.[26] Briefly, SIS was soaked in a solution of 0.4 mg/mL 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (Pierce) and 0.6 mg/mL *N*-hydroxysuccinimide (Pierce) in Activation Buffer (0.1 M 2-[*N*-morpholino]ethane sulfonic acid, 0.5 M NaCl, pH 5.0) for 15 minutes, followed by 2 hours incubation in Coupling Buffer (100 mM sodium phosphate, 150 mM NaCl, pH 7.2). Prior to *in vitro* and *ex vivo* experiments, crosslinked SIS (cSIS) was thoroughly washed with Tris-buffered saline (TBS).

To construct tubular devices suitable for use in the arteriovenous shunt, SIS was rolled around a 4 mm polytetrafluoroethylene dowel with heat shrink connectors at each end. Larger diameter heat shrink tubing was placed on the outside of the SIS and briefly heated to form a tight sheath. Silicone tubing was then stretched over the internal connectors and the outer sheath to create a smooth luminal connection, and the junction was wrapped with Parafilm® (Curwood) to provide a leak-proof seal (Supplementary Figure 1A).

2.2 Extrinsic pathway activity

The extrinsic coagulation pathway is initiated by tissue factor. Tissue factor activity of SIS was quantified by measuring the ability of SIS to catalyze the conversion of Factor X (FX) to activated Factor X (FXa) in a solution containing activated Factor VII (FVIIa). SIS was incubated with 20nM FVIIa and 200nM FX (Enzyme Research Laboratories) in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} for 1 hour at 37°C. The reaction was quenched with ethylenediaminetetraacetic acid (EDTA, 15 mM), and the concentration of FXa was quantified using the chromogenic substrate Spectrozyme® FXa (American Diagnostica) by measuring absorbance at 405 nm for 20 minutes and comparing to known concentrations of FXa.

2.3 Platelet-poor plasma coagulation

Citrated, platelet-poor plasma was collected from 5 juvenile nonhuman primates (*Papio anubis*) and pooled. The diagnostic assays most-commonly used for assessing biomaterial hemocompatibility are the prothrombin time (PT) and the activated partial thromboplastin time (APTT), which measure extrinsic and intrinsic pathway-dependent coagulation, respectively (graphical summary in Supplementary Figure 2). These assays typically

measure coagulation mechanically by monitoring a stainless steel ball bearing in a rotating cuvette containing platelet-poor plasma and the pro-coagulant reagent. The time of coagulation is determined when the ball bearing is displaced due to fibrin polymerization entrapping the bearing. To utilize this testing system, SIS was cut into rings using 2 biopsy punches (outer = 8 mm, inner = 4 mm) and placed at the bottom of the testing cuvettes. The ball bearing, plasma, and pro-coagulant reagent, either Innovin® (Dade®) for the PT or HemosIL® (Instrumentation Laboratories) for the APTT, were then added on top of the SIS and the time to coagulation was automatically measured using a KC1 Delta™ (Tcoag).

The concentration of pro-coagulant stimuli in the PT and APTT reagents are much higher than physiological concentrations. For instance, the PT assay requires >50% inhibition or depletion of extrinsic pathway factors to result in an abnormal clotting time [27]. Therefore, to permit the use of lower concentrations of pro-coagulant stimuli and correspondingly longer clotting times, coagulation was measured optically using a plate reader.[28] Pooled plasma was placed into wells containing SIS, cSIS or no SIS for 15 minutes at 37°C prior to the initiation of coagulation by the addition of an equal volume of 25 mM CaCl₂. The absorbance at 405 nm was measured every 30 seconds, and the clotting time was determined as the time at which the absorbance increased 20% from the initial baseline. Absorbance measurements were stopped at 45 minutes. Clinically, the seminal test to discern whether prolonged clotting is due to an inhibitor or coagulation factor deficiency is to mix the sample plasma with reference plasma 1:1 and perform the APTT. Complete correction of prolonged clotting times is diagnostic of a factor deficiency.[29] Therefore, to determine whether SIS-mediated anticoagulant effects were caused by the presence of an anticoagulant or by coagulation factor depletion via adsorption, SIS-conditioned plasma was mixed 1:1 with native plasma and both the APTT as well as the clotting times following recalcification were measured. In addition to measuring coagulation following recalcification, coagulation was actively stimulated with Innovin that had been diluted in 25 mM Ca²⁺ yielding an estimated final tissue factor concentration of 0.1 pM [30–32], mixed in a ratio of 2:1 reagent to plasma. Coagulation was also stimulated with the addition of 1 nM α-thrombin (Haematologic Technologies, Inc.) to the 25 mM calcium chloride solution. To determine the contribution of contact activation of the intrinsic pathway to SIS-mediated clotting, clotting times were also measured after plasma had been treated with 50 µg/mL corn trypsin inhibitor, which specifically inhibits contact activation.[33] Fibrin polymerization independent of thrombin activation was measured by adding recombinant batroxobin (ProSpec Bio, final concentration 100 ng/mL) to the CaCl₂ solution used to recalcify the plasma.

2.4 Glycosaminoglycan digestion

A number of glycosaminoglycans (GAGs) with anticoagulant properties are known to be present in SIS[7]; however, the relative contribution of these GAGs to the anticoagulant quality of SIS is unknown. To determine the roles of the various GAGs in SIS-mediated anticoagulation, enzymes were used to selectively remove GAGs and characterize the effect on the plasma clotting time. SIS was treated with enzymes that act on hyaluronic acid (hyaluronidase, Sigma), dermatan sulfate (chondroitinase ABC, Sigma), heparin (heparinase I from *Flavobacterium heparinum*, Sigma), and heparan sulfate (heparinase III from

Flavobacterium heparinum, Sigma) according to the conditions listed in Supplementary Table 1. Following enzymatic removal of the GAGs, the treated SIS was incubated in plasma for 15 min, and conditioned plasma was recalcified with an equal volume of 25 mM CaCl₂ to initiate coagulation. Clotting times of plasma samples lacking SIS were used as normalization controls for enzymatic treatments.

2.5 Thrombus formation in a whole blood arteriovenous shunt

Male baboons (*Papio anubis*) used in this study were cared for at the Oregon National Primate Research Center (ONPRC). Experiments were approved by the ONPRC Institutional Animal Care and Use Committee according to the guidelines of the NIH “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care & Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (International Standard Book, Number 0-309-05377-3, 1996).

Tubular SIS devices 4 mm in diameter and 7 cm in length were connected to a baboon arteriovenous femoral shunt (Supplementary Figure 1B) in the absence of anti-platelet or anticoagulant therapies.[34] Autologous platelets and allogenic fibrinogen were radiolabeled with ¹¹¹In and ¹²⁵I, respectively, and infused into the baboon prior to the shunt study. Blood flow through the SIS device was held constant at 100 mL/min using a clamp downstream of the device. The shunt loop was located above a gamma camera (GE 400T gamma scintillation camera with Nuquest® software) to enable the real-time measurement of platelet accumulation using 5 minute exposure times over the course of the 60 minute shunt study. Following the shunt study, the grafts were fixed in 10% formalin (Fisher Scientific) and stored at 4°C until the ¹¹¹In had decayed >10 half-lives, at which point the fibrinogen deposition on the SIS devices was measured using a WIZARD automatic gamma camera (PerkinElmer). Platelet accumulation and fibrinogen deposition were measured on the central 2 cm of the graft to eliminate the potentially confounding effect of increased platelet thrombus formation at the SIS/tubing junctions.

2.6 Statistics

All data are reported as the mean ± standard deviation. A one-way ANOVA with Tukey’s *post hoc* was used to determine significant differences between groups in the *in vitro* plasma coagulation experiments. A student’s t-test was used to determine significant differences between SIS and cSIS in the *ex vivo* whole blood shunt studies. Differences between groups were considered statistically significant if $p < 0.05$.

3. Theory

The biomaterial small intestinal submucosa has been used for vascular applications with very minimal reports of thrombotic complications. The molecular mechanisms responsible for the observed hemocompatibility are currently not well-defined. SIS contains multiple glycosaminoglycans that are known to exert anticoagulant effects by accelerating serpin-mediated inhibition of coagulation factors in the intrinsic and common coagulation pathways. This study uses multiple coagulation assays, spanning from purified systems to a whole blood *ex vivo* shunt loop, to determine the predominant coagulation pathways

specifically inhibited by SIS. By using a variety of model systems, this work provides a thorough characterization of the thrombotic response to SIS, which complements the existing work describing the inflammatory and immunologic responses to the material when used for soft tissue applications.[10,11] Furthermore, this work defines the negative effects of carbodiimide-mediated crosslinking, a treatment commonly applied to SIS, on the hemocompatibility of this biomaterial. Knowing the mechanisms by which SIS biomaterials inhibit thrombus formation, as well as identifying the detrimental effect of crosslinking SIS on hemocompatibility, enables the future development of material modifications, including cell capture strategies and protein linkages which alter local cellular responses and coagulation factor activation.

4. Results

4.1 SIS does not exhibit measurable tissue factor activity

Innovin®, a source of recombinant tissue factor, activated FX in a concentration-dependent manner as expected (Figure 1A). In contrast, SIS generated no measurable amount of FXa and was not significantly different from the samples lacking Innovin® ($p>0.05$, one-way ANOVA). Plasma treated with corn trypsin inhibitor to block contact activation of the intrinsic pathway had a clotting time that was similar whether in the presence or absence of cSIS (Figure 1B), further demonstrating a lack of tissue factor activity in SIS.

4.2 SIS, but not cSIS, inhibits platelet poor plasma coagulation

The PT and APTT are clinical diagnostic assays used to monitor the extrinsic and intrinsic coagulation pathways, respectively. Prolongation of either time indicates a deficiency or inhibition of the respective pathway. Using the standard diagnostic dose of Innovin® for the prothrombin assay, there were no significant differences between native plasma only and plasma with either SIS or cSIS rings in the cuvettes (Figure 2A). In contrast, the APTT was significantly prolonged by SIS compared to plasma only, with a difference between the mean times of 14.3 ± 1.54 seconds (Figure 2B). Crosslinking the SIS abrogated APTT prolongation.

Because of the high concentration of procoagulant stimuli in the APTT and PT reagents, blood clotting times were measured optically to permit lower coagulation stimuli and longer coagulation times. After incubation with SIS for 15 minutes at 37°C, pooled plasma would not coagulate when recalcified (Figure 3A). Crosslinking the SIS abrogated this anticoagulant effect. When plasma was stimulated to coagulate with the addition of 1 nM thrombin, plasma incubated with SIS would not coagulate; in contrast, plasma incubated with cSIS coagulated normally (Figure 3B). Recombinant batroxobin, a thrombin-like serine protease derived from the venom of *Bothrops atrox*, causes fibrin polymerization similar to thrombin, but unlike thrombin, it is not prone to inactivation by antithrombin or heparin cofactor II.[35] Plasma with either SIS or cSIS did not alter the time to coagulation when batroxobin was used to stimulate coagulation (Figure 3C). Prolonged clotting times can be due to coagulation factor deficiencies, due to adsorption on the biomaterial, or an anticoagulant effector. Mixing factor-deficient plasma 1:1 with normal plasma restores coagulation times to normal levels. Conversely, if an anticoagulant is present in plasma,

mixing with normal plasma will not restore coagulation times. Mixing SIS-conditioned plasma 50:50 with native plasma did not restore coagulation times to normal when plasma was recalcified or stimulated with APTT reagent (Figure 4), indicating the anticoagulant effect is mediated by a factor released from the SIS rather than coagulation factor depletion resulting from adsorption to the SIS.

4.3 Crosslinking SIS increases platelet accumulation and fibrinogen deposition

To examine thrombus formation on SIS using flowing whole blood, tubular SIS devices were constructed and connected to an *ex vivo* baboon arteriovenous shunt loop. The number of platelets that accumulated on the central 2 cm of the SIS devices peaked at 35 minutes at $4.16 \pm 1.36 \times 10^8$ platelets per cm^2 (Figure 5A), and reduced to $2.49 \pm 0.52 \times 10^8$ platelets per cm^2 at 60 minutes. In contrast, platelet accumulation continued to increase on cSIS devices for duration of the study, concluding with $9.43 \pm 1.35 \times 10^8$ platelets per cm^2 at 60 minutes. The average amount of fibrinogen deposited onto the central 2 cm of the SIS devices was 105 μg , which was significantly lower than the cSIS devices that had an average of 628 μg of fibrinogen (Figure 5B).

4.4 Heparinase I and heparinase III treatment, but not chondroitinase or hyaluronidase treatment, reduces SIS anticoagulant activity

SIS contains a variety of glycosaminoglycans; notably, a number of these confer anticoagulant activity by increasing serpin-mediated inhibition of FXa and/or thrombin. [36,37] To identify the molecular constituents of SIS responsible for anticoagulant activity, SIS was digested with various enzymes to selectively remove various glycosaminoglycans. Following digestion with the enzymes heparinase I and heparinase III, SIS no longer prolonged plasma coagulation on SIS (Figure 6), resulting in clotting times similar to native plasma and cSIS-treated plasma. However, treatment with chondroitinase ABC (capable of digesting dermatan sulfate) or hyaluronidase did not inhibit SIS anticoagulant activity.

5. Discussion

The decellularized matrix SIS has been utilized as a biomaterial scaffold for many different applications. The prevalent use of SIS in soft tissue reconstruction has resulted in the extensive study of the inflammatory and immune responses to SIS. Carbodiimide-mediated crosslinking of SIS has also been performed to alter the mechanical properties of the material, augment the degradation rate, and functionalize the material with biological molecules.[15,25,38] Recently, SIS has been incorporated into cardiovascular tissue engineering and devices, and in at least one of these cases SIS was carbodiimide-crosslinked.[19] Gross analyses of SIS-based vascular grafts have indicated a lack of thrombus formation.[17,18] To determine the mechanisms of these observations, this work provides a comprehensive characterization of SIS interactions with blood and blood components that utilizes multiple pro-coagulants to stimulate plasma coagulation, including flowing whole blood absent of anticoagulants, and additionally identifies heparin and heparan sulfate as the predominant anticoagulant effectors. This work agrees well with the existing literature on SIS-based cardiovascular devices and provides a more mechanistic

understanding of the ability of SIS to inhibit thrombus formation and the effect of carbodiimide-mediated crosslinking on hemocompatibility.

In native vessel hemostasis and thrombosis, it is generally considered that the extrinsic pathway is the initiator of thrombus formation via tissue factor expression on disturbed vascular endothelium.[39] The extrinsic pathway is responsible for the initial generation of a small amount thrombin, which subsequently triggers local platelet activation. SIS demonstrated a lack of tissue factor activity (Figure 1), defined as the ability to activate FX in a solution of FVIIa and FX. Additionally, cSIS failed to accelerate coagulation when the contact activation was inhibited, further supporting a lack of tissue factor activity. This agrees with past work, showing that tissue factor is generally associated with cells rather than the extracellular matrix[40], and indicates that tissue factor was not released into the SIS during the decellularization process. *In vivo*, once a small amount of thrombin is generated, the intrinsic pathway then becomes the dominant means of FXa and thrombin generation.[41] However, because biomaterials, such as SIS, lack tissue factor and have surface properties different from that of the native vascular endothelium, the intrinsic contact activation pathway may play a more dominant role in biomaterial-mediated thrombosis, though this is controversial.[24,42] The PT and the APTT are two widely-used assays used to identify deficiencies of the extrinsic and intrinsic coagulation pathways, respectively. Using the standard diagnostic concentration of Innovin® (recombinant, lipidated tissue factor), the PT was not affected by the inclusion of SIS or cSIS (Figure 2). In contrast to the PT, inclusion of SIS significantly prolonged the APTT by 14.3 ± 1.54 seconds, indicating inhibition of the intrinsic coagulation pathway. Conversely, cSIS did not significantly alter the APTT. Digestion of the SIS with enzymes that target various GAGs demonstrated that removal of heparin, but not dermatan sulfate or hyaluronic acid, eliminated the anticoagulant effect of SIS. This result is consistent with a prolongation of the APTT, as this assay is known to be particularly sensitive to heparin anticoagulation.[27]

The supra-physiological concentrations of pro-coagulant stimuli in the PT and APTT assays enable rapid testing for the clinical setting; however, it also greatly reduces the sensitivity to anticoagulants and factor deficiencies, as evidenced by the need for extrinsic and common pathway coagulation factors (VII, X, V, prothrombin) to be below 50% of normal to result in a prolonged PT.[27] Therefore, fibrin polymerization was measured optically using low concentrations of pro-coagulant stimuli to initiate coagulation of plasma in SIS-containing wells.[28] When plasma coagulation was initiated by recalcification, as well as when stimulated by the pro-coagulant effectors thrombin (1 nM) or diluted Innovin® (~0.1 pM final tissue factor concentration), plasma with SIS had significantly prolonged coagulation times compared to native plasma (Figure 3). Thus, optical measurement of coagulation revealed that SIS can inhibit tissue factor-initiated coagulation at low tissue factor concentrations but not at the higher concentrations used in the clinical hemostasis assays (i.e. Figure 2). This result is consistent with heparin anticoagulant activity which acts on common pathway coagulation factors (FXa and thrombin) to inhibit coagulation, and also indicates a threshold where high concentrations of tissue factor can overcome this anticoagulant activity. The clinical definition of a coagulation factor deficiency is when a prolonged APTT can be corrected by mixing the patient's plasma 1:1 with a standard

reference plasma.[29] Conversely, failure to restore the APTT to normal indicates the presence of an inhibitor. Because a 1:1 mixture may not be sensitive to detect weak or low concentrations of inhibitors, a 4:1 ratio of patient to standard plasma may be used and the mixed plasma may be incubated for up to 120 minutes to permit inhibitor activity.[43] In this work, SIS conditioned plasma was mixed 1:1 with normal plasma and used immediately, thus using the least sensitive diagnostic conditions to detect coagulation inhibitors. This procedure resulted in a significantly prolonged APTT (Figure 4), indicating the presence of a potent anticoagulant.

Thrombus formation is a dynamic process in whole blood that depends on the transport of cells and factors to the developing thrombus. Flowing blood is therefore necessary to account for the transport dynamics of thrombogenesis as well as shear-dependent platelet accumulation.[44] The *ex vivo* baboon arteriovenous shunt allows whole blood absent of anticoagulant or anti-platelet agents to flow across devices in a controlled manner. After 1 hour of blood flow, SIS devices demonstrated significantly lower platelet accumulation and fibrinogen deposition than cSIS devices. This result corresponds well with the *in vitro* coagulation assays performed in this study, as well as the existing literature that describe minimal thrombus formation on SIS vascular grafts.[17,18] Additionally, the SIS grafts averaged a peak platelet accumulation at 35 minutes, with decreasing numbers of adhered platelets from 40–60 minutes. This result suggests fibrinolysis and loss of fibrin-bound platelets occurring during this time, and suggests minimal thrombus buildup on the devices at longer time periods. In contrast, platelet accumulation on cSIS devices continued to rise throughout the duration of the study, though the rate of accumulation had slowed by 60 minutes (Supplemental figure 3). Similar to the platelet accumulation results, fibrinogen deposition at 60 minutes was also significantly higher on cSIS grafts than on SIS grafts. Platelet accumulation on cSIS per unit surface area is similar to previous results with expanded polytetrafluoroethylene (ePTFE) vascular grafts with 4 mm internal diameter ($0.943 \text{ platelets} \times 10^9/\text{cm}^2$ in this study vs. $\sim 0.87 \text{ platelets} \times 10^9/\text{cm}^2$ historically).[45,46] Although no cSIS devices occluded by 60 minutes, the continual increase in platelet accumulation indicates a risk of eventual occlusion.

Ex vivo arteriovenous shunt experiments were performed to characterize thrombus formation on SIS-based devices in flowing whole blood absent of anticoagulant and anti-platelet therapies to simulate the conditions of vascular devices, such as stents and grafts, in which SIS is being employed. Although this model does not directly measure platelet activation, prior work with this model has determined that reduced platelet accumulation in this shunt model is associated with reduced platelet activation. Specifically, clopidogrel at low-dose ($0.2 \text{ mg}\cdot\text{kg}^{-1}$ for 6 days) significantly reduced platelet and fibrinogen accumulation on vascular grafts and stents, and the addition of aspirin therapy ($10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) further decreased platelet accumulation and fibrinogen deposition.[47] These studies demonstrate that platelet and fibrinogen accumulation in this shunt system is sensitive to inhibition of platelet activation by both ADP and thromboxane A_2 . In addition to activation, platelet accumulation in the shunt model is also dependent on platelet adhesion molecule expression[48] and activated protein C levels.[49] Therefore, platelet accumulation in this arteriovenous shunt model provides a metric that is dependent on multiple pathways of

thrombus formation, thus providing a metric of overall thrombogenicity but lacking direct insight on the mechanism or extent of platelet activation. Future studies may investigate the mechanisms of platelet activation and adhesion on SIS and cSIS.

In all experiments in which SIS exerted an anticoagulant effect, crosslinking the SIS with a carbodiimide consistently eliminated the anticoagulant activity. Coagulation times for plasma with cSIS were similar to plasma alone in both clotting time following recalcification as well as recalcification with inhibition of contact inhibition; furthermore, cSIS demonstrated similar platelet accumulation as previous work with the inert material ePTFE. Thus, crosslinking essentially abolished all biological activity of the SIS with regard to blood-material interaction. Carbodiimides react with free carboxyl groups to enable a reaction with hydroxyl groups and form an amide bond. The GAGs have many free carboxyls, and the collagens have both carboxyl and primary amine groups in their molecular structure and thus could participate in the crosslinking. GAGs known to be in SIS, including heparin, heparan sulfate and dermatan sulfate, are all known to exert anticoagulant effects by enhancing serpin-mediated inhibition of thrombin and FXa.[7,36,37] However, only heparinase I and heparinase III treatment of SIS resulted in a reduction in anticoagulant activity, suggesting that heparin and/or heparan sulfate are the dominant anticoagulant GAGs in SIS, and that glycosaminoglycan crosslinking, which may result in entrapment or chemical alteration of the glycosaminoglycan molecules, is the predominant mechanism by which carbodiimide treatment abrogated the intrinsic anticoagulant activity of SIS. The loss of heparin due to washing away of heparin during the crosslinking process is unlikely, as SIS washed overnight in buffer retained anticoagulant activity, though incubation in plasma eliminated anticoagulant activity (Supplementary Figure 4). Plasma is known to have heparinase enzymes [50,51], giving addition support to the hypothesis that heparin/heparan sulfate are responsible for the anticoagulant effects. Whereas heparinase I cleaves heparin and heparan sulfate at a 3:1 relative activity, heparinase III is generally considered to have a high specificity for heparan sulfate[52]; therefore, the reduction in anticoagulant activity following heparinase III treatment suggests heparan sulfate is the dominant anticoagulant GAG.

Prior work has demonstrated crosslinked SIS to have slower cellular infiltration and an increased inflammatory response.[15] Although crosslinking may be advantageous for increasing the mechanical robustness of SIS, the additional effects on hemocompatibility and inflammatory response may ultimately reduce the clinical utility of the modification in cardiovascular applications such as a vascular graft. The detriment of crosslinking to anticoagulant activity may be overcome by the addition of exogenous heparin during the crosslinking procedure.[16] The addition of exogenous heparin or other anticoagulants, as well as other molecules to enable capture of specific circulating cells or enhance endothelialization, are potential modifications to the crosslinking procedure that may overcome the detriments to hemocompatibility. Novel modifications to SIS and cSIS to have more favorable hemocompatibility or re-endothelialization are areas that could be explored for designing SIS-based cardiovascular devices.

6. Conclusions

This study investigated the hemocompatibility of SIS and carbodiimide crosslinked SIS using an array of assays from individual coagulation factor activation, plasma coagulation, and an *ex vivo* shunt with flowing whole blood. These analyses provide a more in-depth characterization of thrombus formation on SIS and cSIS that agrees with and builds upon existing literature describing histological observations of SIS-based cardiovascular devices. Our results indicate that SIS possesses intrinsic anticoagulant activity due to the presence of heparin/heparan sulfate that confers excellent hemocompatibility; however, crosslinking SIS eliminates this anticoagulant activity and facilitates significant thrombus formation on the material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Tissue factor activity of SIS and clotting times of plasma treated with corn trypsin inhibitor

(A) Tissue factor activity was measured by quantifying the ability of SIS to activate FX in a solution of FVIIa and FX. FX activation was compared to known concentrations of Innovin® – a recombinant, lipidated tissue factor. SIS demonstrated no measurable tissue factor activity ($p > 0.05$, one-way ANOVA with Tukey's *post hoc*, $n = 3$). (B) Corn trypsin inhibitor (50 µg/mL) was added to plasma to inhibit the contact activation of the intrinsic coagulation pathway. Regardless of whether corn trypsin inhibitor was present or absent, plasma clotted at a similar time with and without cSIS. "*" indicates significantly different than plasma only, ($p < 0.05$, one-way ANOVA with Tukey's *post hoc*, $n = 3$). Bars reaching the dotted line indicate the samples did not coagulate by the end of the 45 minute (2700 sec) measurement.

Figure 2. Prothrombin time (PT) and activated partial thromboplastin time (APTT) of plasma and plasma with SIS or cSIS

Rings of SIS or cSIS were placed at the bottom of testing cuvettes, and plasma was placed on top of the material. Coagulation was initiated with Innovin® (A), or HemosIL (B).

“N.S.” indicates no significant difference, while “*” indicates a significant difference between groups (one-way ANOVA with Tukey’s *post hoc*, $p < 0.05$, $n = 4$).

Figure 3. Coagulation times of plasma with SIS and cSIS

Plasma was added to well containing SIS, cSIS, or no SIS for 15 minutes at 37°C, and coagulation was initiated either by recalcification with 25 nM CaCl_2 (A), 5 pM thrombin and 25 nM CaCl_2 (B), or recombinant batroxobin (C). cSIS was not significantly different than plasma lacking SIS for any assay ($p > 0.05$, one-way ANOVA with Tukey's *post hoc*, $n=3$). Bars reaching the dotted line indicate the samples did not coagulate by the end of the 45 minute (2700 sec) measurement.

Figure 4. Mixed plasma clotting times

Plasma was diluted 1:1 in TBS and stimulated to coagulate by recalcification with an equal volume of 25 mM CaCl_2 (A), or APTT reagent (B). The time to coagulation for native plasma, plasma that had been preconditioned with SIS, and a 50:50 mixture of the two were recorded. Bars reaching the dotted line indicate the samples did not coagulate by the end of the 45 minute (2700 sec) measurement. “*” indicates a significant difference between groups (one-way ANOVA with Tukey’s *post hoc*, $p < 0.05$, $n = 3$).

Figure 5. Platelet accumulation and fibrin deposition on SIS and cSIS devices

Autologous platelets and allogenic fibrinogen were radiolabeled with ^{111}In and ^{125}I respectively and infused into the baboon prior to the shunt study. Tubular SIS devices were connected to an *ex vivo* baboon arteriovenous shunt loop absent of anticoagulant or anti-platelet therapies. Real-time platelet accumulation was measured using a gamma camera (A), and end-point fibrinogen deposition was measured after the ^{111}In had decayed > 10 half-lives (B). “*” indicates significantly different from SIS devices, $p < 0.5$ using a Student’s T-test, $n = 3-4$.

Figure 6. Plasma clotting time following enzymatic removal of GAGs

After treating SIS with various enzymes to remove glycosaminoglycans, coagulation of SIS-conditioned plasma was initiated with an equal volume of 25 mM CaCl_2 . Bars reaching the dotted line indicate the samples did not coagulate by the end of the 45 minute (2700 sec) measurement. Plasma only, as well as plasma incubated with cSIS, heparinase I-treated SIS, and heparinase III-treated SIS were not significantly different ($p > 0.05$, one-way ANOVA with Tukey's *post hoc*, $n=3$).